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INTRODUCTION

Radio-isotopes have proved to be a very precious tool for biological research; in fact, many important problems have already been solved by their use.

However, although the developing embryo is a very useful material, these isotopes have not been used much in the embryological field. In an embryo, synthesis of new proteins is a continuous process. Territories differentiate at different times, at different rates and with different mechanisms ; to follow the single steps by which determination takes place is the most important task of embryological research. Since during the process of differentiation some territories probably incorporate amino-acids more rapidly, or more specifically than others, the use of labeled amino-acids might lead to profitable results.

Moreover, as the synthesis of specific proteins is under nuclear control, the problem arises as to how nuclear information is transmitted to cytoplasm. Suggestions that RNA molecules are synthesized in the nucleus and, after receiving « information », are transferred to the cytoplasm, have been given much consideration. The cytoplasm in turn influences the nucleus, which also seems to « differentiate » as development progresses.

Another problem which we wish we understood better concerns the synthesis of DNA.

Problems like these are of primary importance not only to embryologists but also to biochemists and biologists ; thus we have dedicated our attention to them.

Our experiments consisted mainly in « feeding » the Ascidian eggs, at different stages of development, with labeled amino-acids or with labeled nucleic-acid precursors. By autoradiographic methods it was then established *if*, *when*, and *where* the labeled substances were incorporated.

METHODS

The eggs of *Phallusia mamillata* (Ascidians) were used. The eggs removed from the oviducts were incubated in labeled solutions ; the length of time varied in different experi-

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ments. In some experiments the eggs were fixed before fertilization ; in other the eggs were fertilized, and fixed only at the desired stage (blastula, gastrula, neurula, tail bud, larva). In some other experiments the eggs were incubated only at definite stages of development and for short periods. In table I are reported the experimental conditions.

The radioactive substances used were ^{14}C-adenine, ^{3}H-thymidine, ^{14}C-phenylalanine, ^{3}H-phenylalanine, ^{35}S-methionine ; concentrations and specific activity are indicated in table I.

TABLE I

Chemicals	Concentration mM	Specific Activity μc/ml	Stages	
			Unfertilized eggs Incubation time	Fertilized eggs
^{14}C-Adenine	0.55	2.5	3-5 h	—
	0.27	1.25	—	(*)
^{14}C-Phenylalanine	0.24	2.5	3-5 h	(*)
^{3}H-Phenylalanine	0.25	12.5	3-5 h	(*)
^{3}H-Thymidine	0.0033	10	3-5 h	(*)
^{3}H-Thymidine	0.033	100	—	(**)
^{35}S-Methionine	—	—	3 h	—

(*) Incubation for 5-45 minutes and up to the following stages: 2, 4, 8, 16 blastomeres and blastula, gastrula and tail-bud.

(**) Incubation for 2 hours from the stages of 2 blastomeres, blastula, gastrula, neurula and tail-bud.

The controls were eggs which developed in non-radioactive solutions of the same strength, and for the same periods of times. After fixation (with acetic alcohol, or ZENKER solution) the eggs, or embryos, which were incubated in radioactive solutions, were imbedded in agar-paraffin, cut in thin sections (6 μ), washed with non-radioactive solutions, stained (pyronin-methyl green or eosin-hemalum), covered with nuclear emulsion (ILFORD G 5) and finally processed. In some cases the sections were treated with ribo- or deoxyribo-nuclease.

It must be borne in mind that, after the washing of the sections with solutions of the non-radioactive precursor, fixation and numerous treatments with water, alcohol etc., only the macromolecular insoluble fabric of the cell remains, and radioactivity detected by autoradiography indicates incorporation of the labeled precursor in these macromolecular components of the cell (proteins, DNA, RNA, etc.).

I. - *TREATMENT WITH ADENINE*

The eggs were treated, at various stages of development and for different time intervals, either with *non-radioactive* or with *radioactive* adenine. The experiments with non-radioactive adenine were done in order to determine whether adenine influences the normal development ; this proved to be useful, as at certain concentrations adenine was found to be toxic.

A) *Treatment with non-radioactive adenine.* — 0.55 mM adenine blocks the development ; at concentrations lower than 0.27, however, development is quite normal. This blocking is not immediate ; the egg segments, but it does not surpass the blastula stage, the blastomeres becoming fused.

Upon cytological observation the nuclei appear spherical, or in quiescent state ; sometimes there are many nuclei in a cell, apparently originating from the fused blastomeres. This result is not new (BIEBER et al., 1952 ; STEINERT, 1951, 1956) ; and was described in the Ascidians by WADDINGTON & MANCUSO (1955).

The explanation of this observation is not easy ; adenine is a normal component of the nucleic acids, which does not mean, however, that it exists as such in the cytoplasm. However, if adenine is present in the cytoplasm, it must be present in very small quantities, and probably in balanced proportions with the other bases. Possibly strong concentrations of adenine modify this balance, disturbing the nucleic acid synthesis, and perhaps blocking their reproduction. It is known, for instance, that adenine has a specific feedback inhibitory action on purine biosynthesis (GOTS & GOLDSTEIN, 1959). The synthesis of ATP might also be inhibited ; in this case the blocking of the segmentation could be related to a deficiency in this free-energy supply.

B) *Treatment with* ^{14}*C-adenine.* — Since the purpose of these experiments was to determine *if, when,* and *where* adenine is incorporated, we used adenine concentrations of less than 0.27 mM. As noted above, the development of the eggs occurs normally at these concentrations.

In order to see if the different morphological components of the egg take up adenine differently, after incubation we centrifuged the eggs. The components are thereby stratified according to their specific weight ; the morphological and chemical constitution of the different layers, as they result from the centrifugation, are known (LA SPINA, 1958, MANCUSO, 1959 a).

1. — The *unfertilized* eggs, even after 5^h of incubation in ^{14}C-adenine, did not show any radioactivity. From this result we must conclude that if adenine enters the egg (as its inhibitory action in higher concentrations suggests) it is not incorporated ; washes during technical treatment eliminate the non-incorporated adenine. This means that the egg before fertilization does not utilize adenine.

2. — No traces of radioactivity were found in the *fertilized* but still unsegmented egg either. Fertilization does not change the situation with regard to the utilization of adenine. Only the « test-cells » are radioactive (Fig. 1).

3. — The situation changes completely in the young *blastula,* in the *gastrula,* and in the subsequent developmental stages ; the sections of these embryos show large quantities of black granules. The adenine seems, in these cases, to be actually incorporated (Fig. 2). The accurate study of the autoradiographs did not show any differential incorporation of adenine in the different cells or territories of the embryo. However, this does not necessarily mean that the different territories

utilize adenine at the same rate ; in fact, in these experiments the eggs made all their development in radioactive adenine solution ; it is not excluded that *short* incubations, at definite stages of development (gastrula, neurula, tail-bud), would show another situation. It must be recalled that MANCUSO (1959 b), working on Ascidians, showed that RNA, in which adenine enters as a constituent, is not equally distributed in the different cells of the developing embryo. At the 16-cell stage the « micromeres » are more rich in RNA than other cells ; at

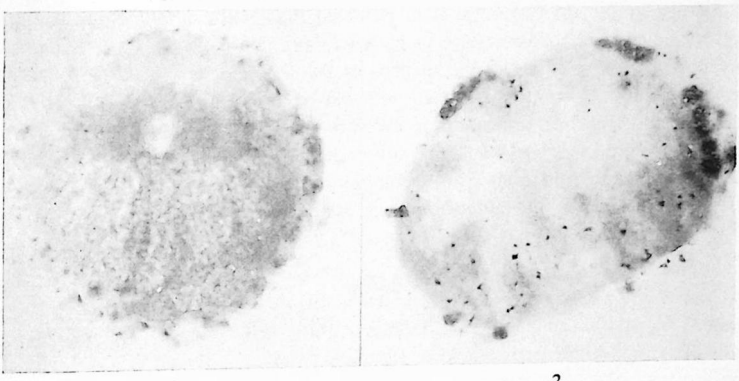

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Fig. 1. — Section of an egg of *Phallusia* autoradiographed after incubation with ^{14}C-adenine for 45 minutes after fertilization. No radioactivity in the cytoplasm.
Fig. 2. — Section of a gastrula of *Phallusia* incubated with ^{14}C-adenine.

the neurula stage, the mesenchyme and neural system also appear richer in cytoplasmic RNA than the other tissues. We expect to obtain more information with the method of short-time incubations, and also with the treatment of sections with ribonuclease ; perhaps in tissues which contain different quantities of RNA the demolition of RNA by the ribonuclease does not occur all at once ; in any case, the treatment with ribonuclease will establish whether the adenine is incorporated in the RNA or in the DNA.

II. - *TREATMENT WITH THYMIDINE*

A) Experiments with non-labeled thymidine show that thymidine does not interfere with the normal development of the eggs. In 0.3 mM thymidine solutions eggs develop at the same rate as the controls, and give rise to swimming tadpoles. Thymidine is a nucleoside ; possibly thymine would have the same toxic action as adenine ; experiments to establish whether the deoxyribose molecule is responsible for the non-toxic action of the thymidine are in course.

B) In the experiments with *labeled thymidine*, ^{3}H-thymidine was used (0.003 mM, specific activity 10 μc/ml ; and 0.03 mM, sp. a. 100 μc/ml).

1. — Several experiments were done on the egg *before* fertilization. The eggs, after removal from gonoducts, were incubated in thymidine solutions for 3-5 hours ; they were then fixed and prepared for autoradiography.

Thymidine is a precursor of DNA : if therefore it is incorporated in the unfertilized egg, it should be found in the egg nucleus. In the Ascidians the nucleus of the egg before fertilization is in the metaphasic stage, at the periphery of the egg, under the cortex. It is to be noted that in the sections of unfertilized egg, we have never found a trace of radioactivity ; consequently, we must exclude any uptake of thymidine by the nucleus of the unfertilized egg. This conclusion, on the other hand, agrees very well with our knowledge of the DNA activity at this stage. In fact, in the metaphasic chromosomes there is no synthesis of DNA ; the two maturation divisions which follow the entering of the sperm are only a mechanism for the distribution of chromosomes already formed ; no new chromosomes are formed in the unfertilized egg at the metaphase.

2. — The situation is different in the *fertilized* egg. Eggs incubated in thymidine solution were fixed from 5′ to 50′ after fertilization. In the fertilized egg the sperm maintains its massive form until after the two maturative segmentations. Up to this moment the sperm is « quiescent », and probably it does not show any metabolic activity ; this situation continues for as long as 30′ after fertilization. Thereafter the nucleus grows, becomes vesicular, and begins those movements which will bring it into the center of the egg, where it meets the egg nucleus and fuses with it. The number of chromosomes when nuclei meet is reconstituted (diploid) ; each chromosome at this stage is longitudinally split or duplicated ; the duplication, which means the reproduction of the DNA, probably happened when the nuclei were still in the vesicular form. This occurs from 30 to 50 minutes after fertilization. The egg then divides.

In the autoradiographs we have not been able to discover any trace of radioactivity until 50′ after fertilization. At this stage there can be seen, in the center of the egg, a cluster of black granules which certainly correspond to the two united nuclei.

We can conclude that the incorporation of thymidine in the egg begins

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Figs. 3-7. — Sections of embryos of *Phallusia* autoradiographed after a short incubation with ^{3}H-thymidine.

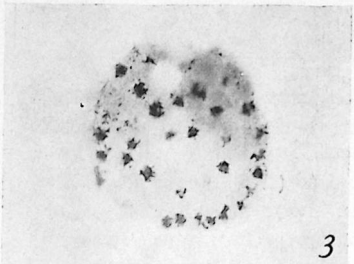
Fig. 3 : neurula with open neural folds.

Fig. 4 : tail-bud stage, sectioned transversally.

Fig. 5 : tail-bud stage. Oblique section.

Figs. 6 and 7 : tail-bud stage. Longitudinal sections.

Abbrev. : *c* = chordal cells ; *e* = ectodermic epithelium ; *i* = intestine primordium ; *m* = muscle cells ; *me* = mesenchyme cells ; *n* = neural tissue.



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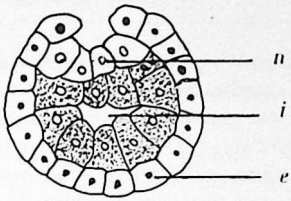

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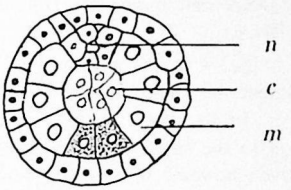

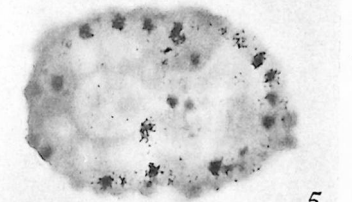

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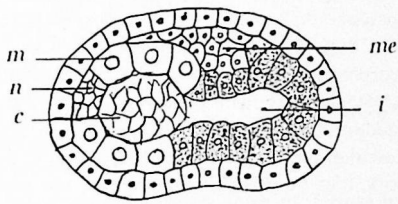

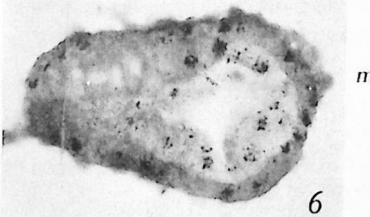

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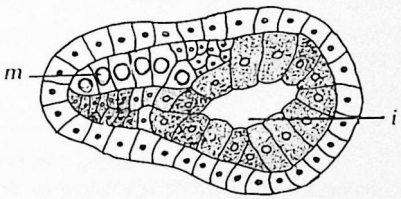

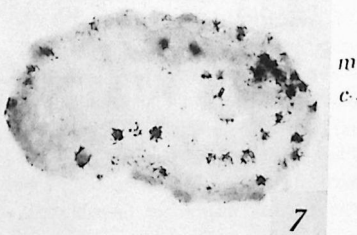

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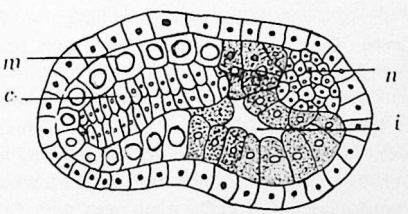

very early ; probably it is taken up by the sperm and egg nuclei in the vesicular or « resting » stage, a few minutes before they meet. There is no reason to suppose that in the Ascidian egg, at the very beginning of development, there is a reserve of DNA in the cytoplasm ; MANCUSO (1959 b), using the Feulgen-reaction, has excluded that possibility. FICQ, PAVAN & BRACHET (1958) also observed that after administration of ^{3}H-thymidine no trace of radioactivity was found in the cytoplasm of the Amphibian oocytes ; and they question whether the DNA determined by HOFF-JØRGENSEN & ZEUTHEN (1952) is really DNA or RNA.

3. — *In the egg at the 2-cell stage* and at the following stages of development the nuclei appear very radioactive.

In the eggs which were incubated in labeled thymidine from the 2-cell stage to the larval stage, we have found that all nuclei in all tissues were radioactive ; however in eggs which were incubated for shorter periods and at definite stages of development (gastrula ; neurula ; tail-bud), not all nuclei were incorporating thymidine. Of particular interest are the aspects of the nuclei of the *neurula*: the nuclei in the closing neural plate are crowded with black spots (fig. 3) ; the nuclei of the mesenchymatic cells also show an active incorporation. The neural and mesenchymatic cells, in fact, multiply, at this embryonic stage, with great rapidity. At the *tail-bud* stage, the entodermic, neural, entodermic and mesenchymatic nuclei are also very radioactive ; the chordal and muscular nuclei, on the contrary, do not show any radioactivity (figs. 4-7). We think that this result is important ; the method of incubating embryos for short intervals enables us to establish the exact moment in which the nuclei or DNA of the different tissues become active. In a tail-bud embryo the muscular and chordal cells no longer segment ; they have no further activity except differentiation. The absence of radioactivity is associated with the loss of the capacity to multiply.

III. - *TREATMENT WITH PHENYLALANINE*

A) *Non-labeled* phenylalanine does not interfere with a normal development of the eggs even in strong concentrations (0.25-0.50 mM).

B) In these experiments ^{14}C-phenylalanine was used at 0.24 mM, specific activity 2.5 µc/ml ; or ^{3}H-phenylalanine at 0.25 mM, sp. a. 12.5 µc/ml.

1. — A number of experiments were concerned with eggs *before fertilization*. They were incubated from 3 to 5 hours, then they were centrifuged, fixed and prepared for autoradiography.

The study of the autoradiographs made it very evident that phenylalanine is taken up by the egg. The four layers in which the cytoplasmic material was distributed by centrifugation did not, however, show an equal uptake. The layers which are hyaline *in vivo*, and which are rich in RNA and consist of ergastoplastic reticulum when seen with the electron microscope (unpublished),

show an intense activity ; the yolk layer shows only a slight activity ; the « test-cells » are very radioactive: these are normally situated all around the egg, but, by centrifugation, are brought to the centripetal pole (fig. 8).

2. — Another group of experiments is concerned with the *eggs which developed* in labeled phenylalanine. Embryos at different stages were taken out of the radioactive solution, fixed and prepared for autoradiography. The study of the autoradiographs showed an equal distribution of black spots in different

Fig. 8. — Section of an unfertilized egg, autoradiographed after incubation for 5 hours with ^{3}H-phenylalanine and centrifuged. Centripetal pole (with test-cells) up.

territories. The experiments were carried out in such a way that it was not possible to check whether some tissues take up more phenylalanine than others ; experiments with shorter incubation periods perhaps will give an answer to this important problem.

IV. - *TREATMENT WITH ^{35}S-METHIONINE*

Only a few experiments were done (on *Ciona* eggs) with ^{35}S-methionine, with the purpose of finding out whether methionine is incorporated in the egg before fertilization.

The eggs were incubated for some hours in ^{35}S-methionine and then the cytoplasmic components were stratified by centrifugation. The autoradiographs showed intense radioactivity. The test-cells appeared to be the most active. Incorporation of ^{35}S-methionine in *Ciona* eggs was already demonstrated in homogenates by Ceas (1959).

DISCUSSION

From the data related above one can draw some general conclusions.

1. — Adenine is a normal precursor of nucleic acids, ATP, and some coenzymes. If, however, we « feed » the eggs at the first stages of development with labeled adenine, the radioactivity is not detected by our autoradiographic technique. It is doubtful whether adenine enters the egg at all. In any case it is not incorporated into the insoluble macromolecular components of the cell. This fact can be explained by supposing that the unfertilized egg is already endowed with a sufficient « charge » of nucleic acid to face the first stages of development. When the « charge » is consumed, new molecules would be synthesized with the incorporation of adenine.

This happens *from the blastula stage* on. Our experiments did not show any differential uptake of adenine in different tissues or preferential uptake by either the cytoplasm or the nucleus. The results do not, however, exclude such a possibility. The method of incubating the eggs for long periods is not the most appropriate one ; we hope to bring some light on this remarkable problem with shorter incubation periods, and the use of ribo- or deoxyribo-nuclease or both ; it should be possible by means of these enzymes to distinguish adenine incorporated in DNA or in RNA.

2. — Thymidine, on the contrary, is taken up from the very first stages of development, and is incorporated into DNA wherever there is nuclear multiplication. It is interesting to note that thymidine is not used for DNA by the unfertilized egg, nor by the fertilized egg during the maturation division, but it is incorporated into nuclear material immediately before the first segmentation and during the following mitotic cycles. This seems to indicate that no cytoplasmic stock of DNA is necessary for the rapid multiplication of the nuclei during the segmentation of the egg. In the embryos at the tail-bud stage the muscular and chordal cells no longer reproduce and their nuclei do not show any radioactivity. Thymidine enters exclusively in the DNA ; possibly it is only involved in the multiplicative activity of the nucleus. The nucleus, however, does not perform this activity only, on the contrary, it is also responsible for the transfer of specific « information » to the cytoplasm. It is generally assumed that this transfer is brought about by means of RNA-molecules. It would be most interesting to follow the steps of this transfer of information up to building of specific proteins.

3. — With regard to the uptake of phenylalanine and methionine, it has resulted from these experiments that the egg, even before fertilization, is capable of taking them up ; at that time it has a proteic metabolism.

The incorporation of the two amino-acids continues during the entire development. All territories are implicated in this uptake ; whether different territories take up different quantitaties of the same amino-acid, or whether one amino-acid is taken up in preference to another, does not results from our analysis.

CONCLUSIONS

1. — Ascidians eggs were treated, at different stages of development and for different time intervals, with labeled precursors of nucleic acids (^{14}C-adenine, ^{3}H-thymidine) or with labeled amino-acids (^{14}C-phenylalanine, ^{3}H-phenylalanine, ^{35}S-methionine).

The eggs were then treated by autoradiographic methods.

2. — *Non-labeled* adenine, at certain concentrations, is toxic for the eggs, which are blocked at the blastula stage; at lower concentrations, however, the development occurs normally. Labeled adenine at non-toxic concentrations also permits normal development; adenine is incorporated from the blastula stage; it is not incorporated earlier.

3. — Thymidine is not incorporated in the unfertilized egg; the fertilized egg begins to incorporate it only a few minutes before segmentation. This uptake is strictly limited to the nuclei, and one can assume that the intensity of incorporation is proportional to the multiplicative activity of the DNA. With short incubation periods in tritiated thymidine solution, in a young larva only the nuclei of the growing tissues show a very high activity; the nuclei of chordal or muscular cells, which no longer divide, do not show any radioactivity.

4. — ^{14}C-phenylalanine, ^{3}H-phenylalanine and ^{35}S-methionine are taken up by the eggs from the stage preceding fertilization. In the case of phenylalanine this uptake continues until the late stages of development.

RIASSUNTO

1. — Uova di Ascidie furono trattate, a differenti stadi e per tempi diversi, con precursori marcati di acidi nucleici (^{14}C-adenina, ^{3}H-timidina) o con amino-acidi marcati (^{14}C-fenilalanina, ^{3}H-fenilalanina, S^{35}-metionina).

Le uova furono poi trattate con metodi autoradiografici.

2. — L'adenina non marcata, a determinate concentrazioni, è tossica per le uova, che sono bloccate allo stadio di blastula; a concentrazioni più basse, tuttavia, lo sviluppo ha luogo normalmente. Usando adenina marcata a concentrazioni non tossiche, si osserva che questo composto è incorporato a partire dallo stadio di blastula, ma non è incorporato negli stadi più precoci.

3. — La timidina non è incorporata nell'uovo vergine; l'uovo fecondato comincia ad incorporarla solo pochi minuti prima della segmentazione. Questa incorporazione è strettamente limitata al nucleo e si può ritenere che l'intensità di incorporazione sia proporzionale all'attività moltiplicativa del DNA. Dopo periodi brevi di incubazione in soluzioni di timidina tritiata, nella giovane larva solo i nuclei dei tessuti in accrescimento mostrano un'attività molto elevata, mentre i nuclei delle cellule cordali o muscolari, che non si dividono più, non mostrano alcuna radioattività.

4. — ^{14}C-fenilalanina, ^{3}H-fenilalanina e ^{35}S-metionina sono incorporate dalle uova prima della fecondazione. Nel caso della fenilalanina questa incorporazione continua fino agli stadi più avanzati di sviluppo.

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